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Phosphorylation of Chemoattractant Receptors Is Not Essential for Chemotaxis or Termination of G-protein-mediated Responses*

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In several G-protein-coupled signaling systems, ligand-induced receptor phosphorylation by specific kinases is suggested to lead to desensitization via mechanisms including receptor/G-protein uncoupling, receptor internalization, and receptor down-regulation. We report here that elimination of phosphorylation of a chemoattractant receptor of *Dictyostelium*, either by site-directed substitution of the serines or by truncation of the C-terminal cytoplasmic domain, completely prevented agonist-induced loss of ligand binding but did not impair the adaptation of several receptor-mediated responses including the activation of adenylyl and guanylyl cyclases and actin polymerization. In addition, the phosphorylation-deficient receptors were capable of mediating chemotaxis, aggregation, and differentiation. We propose that for chemoattractant receptors agonist-induced phosphorylation regulates surface binding activity but other phosphorylation-independent mechanisms mediate response adaptation.

A diverse array of hormones, neurotransmitters, and environmental stimuli are sensed by cell surface receptors that transduce the signals to heterotrimeric G-proteins¹ within the cell (1, 2). This extensive family of receptors shares a number of structural and functional features (3). All of the members contain seven transmembrane domains that undergo conformation changes upon ligand binding, revealing G-protein coupling domains in the cytoplasmic loops of the receptors. In this activated state, these receptors promote the exchange of GDP bound to heterotrimeric G-proteins with GTP and, thereby, initiate downstream signaling.

Termination of this signaling is critical in order for cells to respond to graded stimuli. The GTPase activity of the G-protein, assisted in some instances by RGS proteins (regulators of G-protein signaling), terminates the response once the excitatory input from the receptor is removed. In addition, multiple desensitization mechanisms exist that serve to attenuate re-

sponses despite persistent stimulation (4). First, there is a rapid functional uncoupling of these receptors from G-proteins. Second, the ability of receptors to bind ligand can be diminished due to modulation of their affinity (5) or to becoming inaccessible through "sequestration" or internalization. Last, prolonged exposure to ligand leads to an actual reduction of receptor levels (down-regulation), the consequence of accelerated degradation and/or diminished transcription. In the case of β -adrenergic receptors and rhodopsin, uncoupling is attributed to receptor phosphorylation on cytoplasmic C-terminal domain serines/threonines by G-protein-coupled receptor kinases, followed by the stoichiometric association of the phosphoreceptors with arrestins (6). This receptor kinase/arrestin paradigm, however, may not be the only means for response termination as other points in the pathway could be regulated to effect adaptation.

The social amoebae *Dictyostelium discoideum* provides an excellent model system for genetic analysis of G-protein-coupled receptors (7). Starvation initiates a developmental program in which thousands of free living amoebae aggregate and differentiate to form a multicellular structure containing spores. A family of four sequentially expressed G-protein-coupled cAMP receptors (cARs) are essential at distinct stages in development. cAR1 mediates three principal physiological responses during aggregation including chemotaxis, cell-to-cell relay of the cAMP signal, and regulation of gene expression (8). The activation of heterotrimeric G-proteins by cAR1 and the sequence of events following this activation are highly analogous to those occurring in mammalian cells.

Many cAR1-mediated responses exhibit adaptation (9). For example, receptor-mediated activation of adenylyl cyclase reaches a peak in 1 min and then subsides within 5–10 min despite continued stimulation. When the stimulus is removed, the cells regain sensitivity with a half-time of a few minutes. cAMP-induced cell shape changes, actin polymerization, cGMP synthesis, and myosin heavy and light chain phosphorylations also adapt in a similar fashion. However, the kinetics of these responses differ from one another, suggesting the involvement of multiple independent adaptation processes. The requirement for pulses of extracellular cAMP to induce expression of a number of genes during aggregation suggests that the mechanism by which cAR1 regulates gene expression is also subject to adaptation.

Adaptation of many of these responses is closely correlated with cAR1 phosphorylation (10). Increments in stimuli augment cAR1 phosphorylation, which reaches a steady-state level as the responses subside. When the stimulus is removed, the extent of phosphorylation declines and, concomitantly, the cells regain sensitivity. Saturating stimuli result in the modification of 3 to 4 serines in the C-terminal cytoplasmic domain of the

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¹ The abbreviations used are: G-protein, guanine nucleotide-binding regulatory protein; cAR, cAMP receptor; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; DTT, dithiothreitol; Pipes, 1,4-piperazinedithanesulfonic acid; TRITC, tetramethylrhodamine isothiocyanate.

receptor (11). Phosphorylation of either Ser-303 or Ser-304 results in a discrete shift in cAR1's electrophoretic mobility and is highly correlated with an agonist-induced decrease in the receptor's affinity (12).

In the present study, we sought to determine the role of the agonist-induced cAR1 phosphorylation in adaptation of several chemoattractant-mediated processes *in vivo*. Our results have led us to the unexpected conclusion that phosphorylation of cAR1 is not essential for the termination of any of the responses we tested, including agonist-induced adenylyl cyclase activation, actin polymerization, chemotaxis, and gene expression. Therefore, there must be a novel phosphorylation-independent adaptation mechanism (or mechanisms) that attenuate these responses.

EXPERIMENTAL PROCEDURES

Generation of cAR1 Mutants—cAR1 mutant cm1234, generated by site-directed mutagenesis as described previously (13), lacks all 18 serines of the C-terminal cytoplasmic domain. In brief, the 10 serines in clusters 1, 2, and 4 (as defined in Ref. 13) were substituted with either glycine or alanine. The 8 serines of cluster 3 and an intervening leucine were deleted. A second mutant, T289, created by exonuclease III-mediated deletion of a cAR1 cDNA, is truncated after Leu-289, eliminating most of the C-terminal domain including all of its serine residues. Its new C terminus includes the vector-encoded peptide GEEFD. Both mutant cDNAs were subcloned into the G418-selectable, extrachromosomal expression vector pJK1 (14).

Generation, Growth, and Development of Cell Lines Expressing cAR1 Mutants—A *car1⁻/car3⁻* cell line, designated RI9, was derived from JB4 cells (15) in two steps. Each of two cAR1 loci in JB4 cells is, in part, replaced with a selectable *PYR5-6* gene fragment. First, both *PYR5-6* fragments were eliminated by homologous recombination using an internally deleted cAR1 fragment (*i.e.* the predecessor of pMC25; Ref. 15), yielding the *car1⁻* uracil auxotroph, JB5. Finally, cAR3 was disrupted in JB5 cells to yield RI9 cells using the prototrophy-restoring construct 5cAR3ura as described previously (16). All homologous recombination events were confirmed by Southern analysis.

Receptor expression constructs from above were introduced into RI9 cells by electroporation. Clonal transformants were selected for and maintained in HL5 medium (17) supplemented with G418 (20 μ g/ml). Unless noted otherwise, experiments were conducted with cells grown in a shaken suspension to 5×10^6 cells/ml, washed with DB (5 mM KH_2PO_4 , 5 mM Na_2HPO_4 , 2 mM MgSO_4 , 0.2 mM CaCl_2), and developed (*i.e.* starved) for 5 h at 2×10^7 cells/ml of DB with shaking (120 rpm) and pulsatile cAMP addition (50–100 nM) every 6 min.

In Vivo ^{32}P -Labeling and Immunoblotting of cAR1 Mutants—Developed cells expressing wild-type and mutant receptors were labeled for 40 min with carrier-free ^{32}P , as described previously (13). Where indicated, cells were stimulated with 1 μ M cAMP in the presence of 10 mM DTT for the final 10 min. Labeled cells were extracted for 5 min with ice-cold 1% CHAPS in ERB (13) and centrifuged at 4 °C for 10 min at 10,000 rpm in a Sorvall SS-34 rotor. After two additional CHAPS extractions, the pellets that contained cAR1 were solubilized in sample buffer and 10^7 cell equivalents of each were subjected to SDS-polyacrylamide gel electrophoresis on a 15% "low bis" gel (0.073% w/v bis-acrylamide). SDS-polyacrylamide gel electrophoresis-resolved proteins were transferred to a polyvinylidene fluoride membrane and probed with antiserum directed against the peptide sequence KREPEPER-FEY in the second intracellular loop of cAR1 (provided by M. Caterina). Primary antibodies were detected using horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham) and chemiluminescence (DuPont). Residual chemiluminescence was then quenched by washing the blot with 0.02% sodium azide for 15 min, and autoradiography was performed overnight to detect the ^{32}P incorporated into the receptors.

Receptor Binding Analysis—Growth-stage cells were washed with PB (5 mM KH_2PO_4 , 5 mM Na_2HPO_4), resuspended at 3×10^7 cells/ml in PB containing 5 mM caffeine (to prevent spontaneous activation of adenylyl cyclase), and shaken for 30 min at room temperature. Caffeine-treated cells were washed with ice-cold PB and resuspended at a density of 10^8 cells/ml. cAMP binding assays were performed as described before, separating cell bound and unbound cAMP by centrifugation through oil (18). Scatchard data were analyzed using the program LIGAND. For the loss of ligand binding measurements, caffeine-treated and washed cells were further treated with PB alone or with 10

μ M cAMP in the presence of 10 mM DTT for 15 min. The cells were then diluted 20-fold with ice-cold PB, rapidly washed 4 times in the same buffer, and resuspended at 10^8 cells/ml. Their saturable cAMP binding was then measured with 16 nM [^3H]cAMP (15).

Adenylyl Cyclase Assays—The cAMP-induced synthesis and secretion of [^3H]cAMP was assayed by the perfusion method previously described (19). Vegetative amoebae were fed with [^3H]adenosine-labeled bacteria in a shaken suspension and then allowed to develop for 5–8 h on DB agar plates. The cells were then transferred to filters, perfused with DB with or without cAMP at a flow rate of 10 drops/min. Effluent (1 min fractions) was collected into test tubes preloaded with 20 μ l of stop solution (0.2 M HCl, 1 mM cAMP, 50 mM DTT). [^3H]cAMP was isolated by Dowex and alumina chromatography and quantitated by scintillation counting. The adenylyl cyclase "activation trap" assay was performed as described previously (20). Developed cells were treated for 30 min with 5 mM caffeine and washed 4 times with ice-cold PM (5 mM KH_2PO_4 , 5 mM Na_2HPO_4 , 2 mM MgSO_4). The cells (8×10^7 cells/ml) were then stimulated at 22 °C with 10 μ M cAMP and aliquots were lysed at the indicated times. The amount of [α - ^{32}P]ATP converted to [^{32}P]cAMP by each lysate in 1 min was measured.

Guanylyl Cyclase Assay—cGMP accumulation resulting from guanylyl cyclase activation was measured as described previously (21). Cells were developed for 5 h, brought to a basal state by dilution and rapid shaking (20), and then stimulated with 10 nM cAMP or 5 μ M 2'-deoxy-cAMP in shaking culture at 22 °C. At designated times, aliquots of cells were removed and lysed with perchloric acid (final 3.5%). Samples were neutralized with KHCO_3 (50% saturated at 22 °C), and the amount of cGMP was measured by a competitive radioimmunoassay (Amersham).

Actin Polymerization—Developed cells were prepared as described for the adenylyl cyclase activation trap assay, resuspended in PM buffer at 3×10^7 cells/ml. Cells were kept on ice until just prior to experiments, and then they were warmed to room temperature by shaking for 10 min. At time 0, 100 nM cAMP was added to stimulate cAMP-induced actin polymerization *in vivo*. At the indicated times, 100- μ l aliquots of cells were combined with 1 ml of fixing solution (3.7% formaldehyde, 10 mM Pipes, 0.1% Triton X-100, 20 mM $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, 5 mM EGTA, 2 mM MgCl_2 , 250 nM TRITC-phalloidin, pH 6.8), which served to permeabilize the cells, fix, and stain F-actin (22). After staining for 1 h at room temperature, fixed cells were centrifuged (14,000 rpm, 5 min). TRITC-phalloidin was then extracted from the pellet with methanol (1 ml) and quantitated by fluorimetry (540-nm excitation, 575-nm emission).

Chemotaxis Assay—Chemotaxis was measured by the small population assay (23). Cells were developed in shaking culture for 6 h with 100 nM cAMP pulses and diluted to 2×10^6 cells/ml with DB. Droplets (~0.1 μ l) containing 100–200 cells were spotted with a microcapillary tube adjacent to droplets of varying cAMP concentration on 1% agar plates containing DB and 3 mM caffeine. After 10–20 min in a humidified chamber, cell drops were examined for chemotaxis. Chemotactic responses were scored positive if at least twice as many amoebae were pressed against the edge nearest the cAMP droplet compared with the opposite edge.

RNA Isolation and Analysis—For each of the conditions, total cellular RNA was isolated from 2.5×10^7 cells as described by Nellen *et al.* (24), size fractionated on 1.5% agarose gels containing 2.2 M formaldehyde, and transferred to nylon membranes. Northern transfers were hybridized to [^{32}P]dATP-labeled probes according to standard procedures and exposed to x-ray film.

RESULTS

Mutants cm1234 and T289 Fail to Undergo Phosphorylation—To test the role of phosphorylation in regulation of receptor function, we constructed two mutant forms of cAR1. In cm1234, all 18 serines of the C-terminal cytoplasmic domain were either substituted or deleted (13), whereas in T289 the C-terminal 105 amino acid residues were deleted including all of the serines in the cytoplasmic domain. Wild-type and mutant receptors were expressed constitutively under the control of the actin-15 promoter in cells lacking both cAR1 and cAR3 genes. We elected to use *car1⁻/car3⁻* because cAR3 mediates some residual responses to cAMP in the absence of cAR1 (25, 26) and appears to be dispensable (16). Thus, in this genetic background, the measured responses could be attributed entirely to the ectopically expressed receptors. As shown in Fig. 1A, the cells expressed relatively comparable steady-state levels of the respective receptors. However, the expression of T289 was fre-

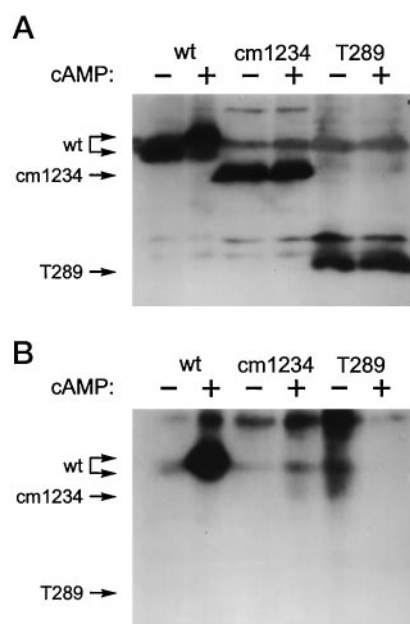


FIG. 1. In vivo phosphorylation of wild-type cAR1 and mutant receptors. Developed cells expressing either wild-type cAR1 (*wt*), cm1234, or T289 were labeled with [32 P]orthophosphate. Unstimulated (–) and cAMP-stimulated (+) samples of each were extracted with CHAPS. The receptors, greatly enriched and quantitatively recovered in the CHAPS-insoluble fraction, were then analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting with anti-cAR1 serum and chemiluminescent detection (panel A). After quenching residual chemiluminescence, cAR1-associated 32 P was detected by autoradiography (panel B). The position of each receptor construct is indicated including the upshifted phosphorylated form of wild-type cAR1. A non-specific band migrates between the two forms of wild-type cAR1 in panel A.

quently reduced to 70% of wild-type cAR1. The molecular weight of cm1234 is lower due to the deletion of 9 residues that encompass one of the clusters of serines. As we have shown previously, the cm1234 receptor does not exhibit the cAMP-induced electrophoretic mobility shift characteristic of the wild-type receptor. Similar results were obtained for T289, which exhibited an apparent molecular mass of 25 kDa (Fig. 1A).

To verify that cm1234 and T289 lack phosphorylation, the cells were metabolically labeled with [32 P]orthophosphate, and its incorporation into each receptor was assessed for both cAMP-stimulated and unstimulated cells. Compared with wild-type cAR1, [32 P]phosphate incorporation into cm1234 or T289 was dramatically reduced; both basal and cAMP-induced phosphorylation were virtually undetectable (Fig. 1B).

These cell lines were used for analysis of the functional consequences of removing phosphorylation sites. We first performed Scatchard analysis on both wild-type and cm1234. As shown in Fig. 2A, the two receptors displayed similar K_d and B_{max} values. T289 also had a similar K_d although often its B_{max} was reduced due to lower expression levels (data not shown). Thus, any observed physiological and biochemical phenotypes conferred by the mutant receptors cannot be attributed to differences in the binding characteristics.

Loss of Ligand Binding Does Not Occur in the Nonphosphorylatable Mutants—Cells expressing wild-type cAR1 exhibit “loss of ligand binding” in response to cAMP treatment. The apparent loss is due to an agonist-induced reduction in the affinity of the cell surface receptors and can be conveniently monitored with a binding experiment at a subsaturating concentration of [3 H]cAMP. This process is highly correlated with phosphorylation of serine residues 303 and 304. This reduction in affinity does not merely reflect uncoupling of the phospho-

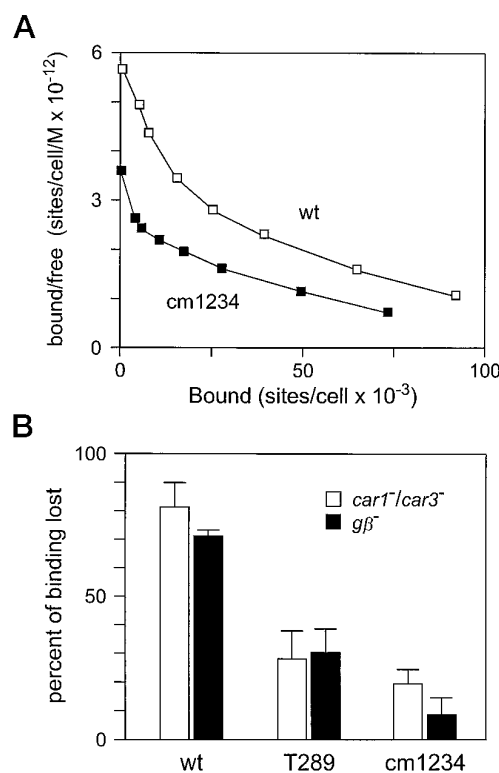


FIG. 2. Ligand binding properties of the nonphosphorylatable receptors. Panel A, Scatchard analysis of cAMP binding. Growth-stage *car1⁻/car3⁻* cells overexpressing either wild-type cAR1 or cm1234 were harvested, treated with caffeine, and resuspended in phosphate buffer. Competition of [3 H]cAMP binding by unlabeled cAMP, ranging in concentration from 10^{-9} M to 2×10^{-6} M, was measured. Bound cAMP was separated from unbound cAMP by pelleting the cells through silicon oil as described previously (18). The means of triplicate determinations are shown. Computer analysis of this data using LIGAND yielded the following two-site models. Wild-type: $K_{d1} = 47 (\pm 11)$ nM; $B_{max1} = 36 (\pm 10) \times 10^3$ sites/cell; $K_{d2} = 608 (\pm 156)$ nM; $B_{max2} = 365 (\pm 25) \times 10^3$ sites/cell; cm1234: $K_{d1} = 30 (\pm 5)$ nM; $B_{max1} = 12 (\pm 5) \times 10^3$ sites/cell; $K_{d2} = 819 (\pm 120)$ nM; $B_{max2} = 292 (\pm 36) \times 10^3$ sites/cell. Panel B, loss of ligand binding of the mutant receptors. The receptors were expressed in *car1⁻/car3⁻* (open bars) and $g\beta^-$ cells (solid bars). cAMP-induced loss of binding was measured as described under “Experimental Procedures.” The reduction of binding due to cAMP pretreatment is expressed as a percentage of the binding exhibited by buffer-treated cells. In the experiment depicted, the buffer-treated *car1⁻/car3⁻* cells expressing wild-type cAR1, cm1234, and T289 bound 2300, 3000, and 1100 cpm/ 8×10^6 cells, respectively. The corresponding $g\beta^-$ transformants bound 2600, 4000, and 1000 cpm/ 8×10^6 cells.

receptor from G-protein as it occurs to a normal extent in cells lacking the G-protein β -subunit, confirming that it is a G-protein-independent phenomenon (Fig. 2B). As shown in Fig. 2B, the response is equally impaired for mutant receptors in the absence of the β -subunit.

Nonphosphorylatable Receptors Are Able to Support Development—We first examined the ability of cm1234 and T289 to support development. As was previously shown, *car1⁻/car3⁻* cells fail to aggregate, and normal development can be restored by introducing a wild-type cAR1 expressed constitutively from actin promoter (8). Remarkably, the cm1234 and T289 cells exhibited a relatively normal progression in development to the aggregation stage. Time-lapse video microscopy of starving cell populations on nonnutrient agar were performed as an indirect means of observing cAMP wave propagation. The influence of cAMP waves on cell shape change is apparent in time-lapse phase contrast images. Whereas waves with periodicity of 6 min were routinely observed with wild-type cells, similarly timed “bursts” were observed near aggregation centers with the nonphosphorylatable cells. However, these waves were

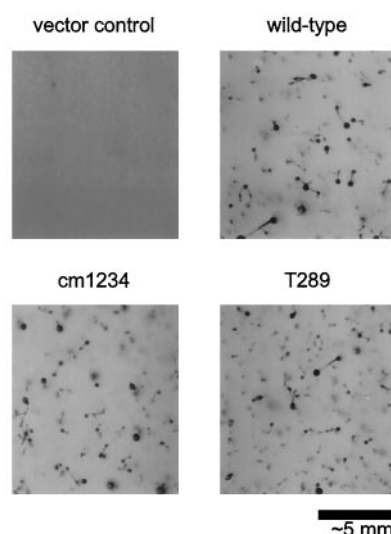


FIG. 3. Development of cells expressing wild-type cAR1 or mutant receptors. *car1*[−]/*car3*[−] cells expressing either wild-type cAR1, cm1234, or T289 were tested for their ability to undergo development. In addition, *car1*[−]/*car3*[−] cells transformed with the empty vector, pJK1, were also included as a control. Growth-stage cells were washed twice with DB and plated (10^6 cells/cm²) on 10-cm nutrient-free plates (1% agar in DB). After allowing cells to settle and adhere for 20 min, excess buffer was drained, and cells were left to develop undisturbed for 2 days at room temperature. The rounded spore masses (often out of the plane of focus) and associated stalks of fruiting bodies are apparent in all but the vector control cells.

rarely propagated as effectively as in wild type (data not shown). This suggests that some receptor-mediated responses may not be completely normal. In addition, when cm1234 or T289 are overexpressed at high levels, arrest of development at the mound stage is observed (data not shown). We are currently investigating the cause of this phenotype. Nevertheless, as shown in Fig. 3, the nonphosphorylatable receptors when expressed at physiological levels can mediate normal development and produce apparently normal fruiting bodies that are comparable to those of the wild-type cAR1 expressing control cells.

Nonphosphorylatable Receptors Mediate Chemotaxis—The ability to aggregate suggests that the mutant receptors are able to mediate chemotaxis. We therefore examined the chemotaxis of cm1234 and T289 cells toward a wide range of cAMP concentrations. As shown in Fig. 4, these cells were capable of chemotaxis with a dose-response profile similar to that of cells expressing wild-type cAR1. The EC₅₀ for chemotaxis by the mutant cell lines is about 2-fold higher than wild-type cells, and at higher cAMP concentration the response by mutant receptors declined faster than wild-type receptors. We also carried out chemotaxis assays on a series of background cAMP concentrations. In these assays, the cells are first placed on agar containing a uniform concentration of attractant. After 15 min, phosphodiesterase secreted by the cells degrades cAMP locally, and an agonist gradient extending away from the spot of cells is established. We observed no differences in the behavior of the cells expressing mutant and wild-type receptors (data not shown). Thus, chemoattractant receptor phosphorylation does not play a critical role in chemotaxis.

Kinetics of Chemoattractant-mediated Actin Polymerization Are Independent of Receptor Phosphorylation—The chemotaxis response mediated by the mutant receptors were similar to that of wild-type receptors. However, there were subtle differences in the maximal response and the width of the dose response curve. So we examined some of the responses closely associated with chemotaxis such as the rapid chemoattractant-

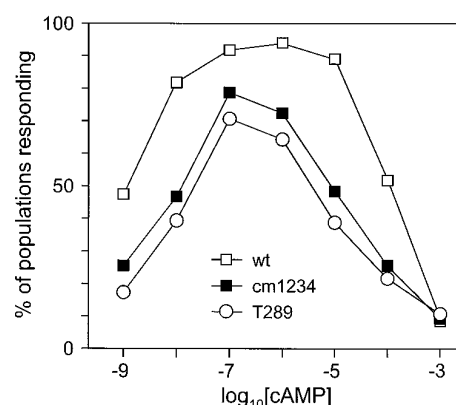


FIG. 4. Chemotaxis to a range of cAMP concentrations mediated by wild-type or mutant receptors. The ability of *car1*[−]/*car3*[−] cells expressing wild-type cAR1, cm1234, or T289 to exhibit chemotaxis in response to a range of cAMP stimuli was examined using the small population assay of Konijn (23). For each cAMP concentration tested, the percentage of populations judged to have responded is plotted versus the logarithm of the molar cAMP concentration.

mediated actin polymerization and activation of guanylyl cyclase. Actin polymerization is involved in the series of transient cell shape changes that occur upon addition of chemoattractant. Typically filamentous actin levels rise 2–3-fold within 6 s and then decrease to nearly base-line levels within 15 s. Concurrently, the cells cease moving and round up. Frequently a second, slower increase in F-actin occurs, peaking at 60–90 s, which coincides with a spreading of the cells on the surface. Finally, F-actin levels return to the prestimulus levels and cells resume their prestimulus randomly motile behavior. We reasoned that if receptor phosphorylation was essential for adaptation of the F-actin response, one or more of the F-actin changes would be delayed in the *car1*[−]/*car3*[−] cells expressing the mutant receptors. However, as illustrated in Fig. 5A, the responses in the wild-type and mutant cells followed very similar profiles and clearly returned to base-line levels within 5 min.

The Kinetics of Guanylyl Cyclase Activation Are Independent of Receptor Phosphorylation—Another G-protein-coupled response mediated by the chemoattractant receptor is transient activation of guanylyl cyclase. As shown in Fig. 5B, stimulation of both wild-type and cm1234 cells with 2'-deoxy-cAMP produces transient activation and accumulation of cGMP. Ten seconds after stimulation, the level of cGMP increases ten-fold returning to the basal level after 45 s. The responses mediated by wild-type and mutant receptors are indistinguishable, suggesting that in addition to the adenylyl cyclase and actin polymerization responses, the guanylyl cyclase response adapts as well.

Kinetics of Chemoattractant-mediated Adenylyl Cyclase Activation—The ability of the cells to aggregate also suggested that cell-cell signaling, which depends on appropriate regulation of adenylyl cyclase, is normal. To directly assess the signaling response, an analysis of cAMP-induced cAMP secretion was performed on the cm1234 and T289 cells. As previously documented, the time-course of cAMP secretion directly reflects the state of activation of adenylyl cyclase. As shown in Fig. 6A, the kinetics of secretion of cAMP from the cells expressing either the T289 or cm1234 were nearly identical to those of cells expressing the wild-type receptor. In all cases, cAMP elicited a transient increase in the rate of cAMP secretion that peaked after 2 min and subsided after 10 min of stimulation. In eleven similar experiments, there were no significant differences in the rate of decline in secretion between mutants and wild-type cells (Fig. 6A).

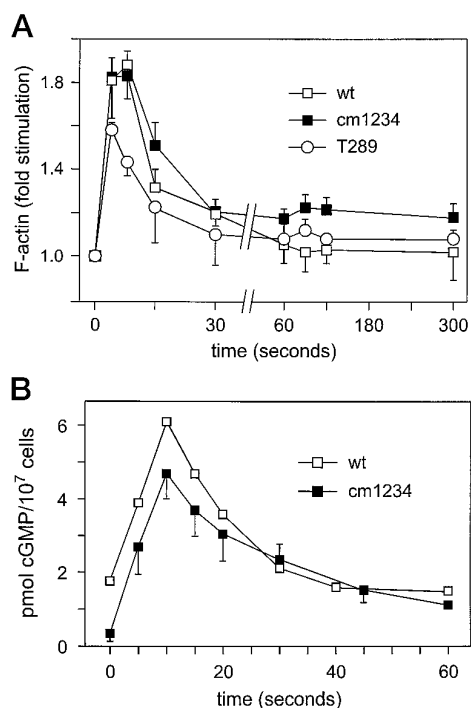


FIG. 5. Chemoattractant-induced actin polymerization (A) and guanylyl cyclase activation (B). Both cAMP-induced responses were measured in cells developed for 5 h. *Panel A*, *car1*[−]/*car3*[−] cells expressing wild-type cAR1, T289, or cm1234 were stimulated with 100 nM cAMP and 10 mM DTT. At the indicated times, aliquots of cells were transferred to a quenching solution to simultaneously stop the reaction and stain filamentous actin with TRITC-conjugated phalloidin. A measured amount of stimulated F-actin was normalized to the amount at the basal state and a relative amount of polymerized actin is plotted. *Panel B*, cGMP accumulation in *car1*[−]/*car3*[−] cells expressing cm1234 in response to stimulation with 5 mM 2'-deoxy-cAMP and 5 mM DTT is compared with that in wild-type AX3 cells stimulated with 10 nM cAMP.

We also measured the catalytic activity of adenylyl cyclase after cAMP stimulation in an activation trap assay. For this assay, cells were stimulated with cAMP for varying lengths of time and catalytic activity was measured immediately after lysis. As shown in Fig. 6B, the profile of activation and inactivation of the enzyme was indistinguishable between wild-type cells and the mutants. We also measured the total production of cAMP from cm1234 cells in suspension following stimulation with 2'-deoxy-cAMP, which was also transient (data not shown). Thus, under the conditions tested, the two mutant receptors appear to elicit kinetically identical responses. Therefore, there must be a cAR1 phosphorylation-independent mechanism of adaptation for this response.

Phosphorylation Is Not Required for cAMP-dependent Gene Expression—In addition to mediating aggregation, repeated cAMP stimuli are required for induction of a class of genes expressed in early aggregation. Treatment of cells with constant levels of cAMP, at low or high concentrations, suppresses expression of these genes. This requirement for pulsatile stimulation suggests that the pathway that leads to expression of these genes depends on a response that adapts. To determine the role of cAR1 phosphorylation in this adaptation process, we examined the induction of mRNA encoding the cell adhesion protein, contact site A in *car1*[−]/*car3*[−] cells expressing cm1234. As was observed with the wild-type receptor, cm1234 mediated the accumulation of contact site A mRNA in response to intermittent cAMP stimuli and also repressed its expression in response to constant cAMP (Fig. 7). The pulsatile addition of cAMP at 6-min intervals was an effective inducer at 30, 300, and 900 nM, whereas continuous fluxes of the nucleotide at

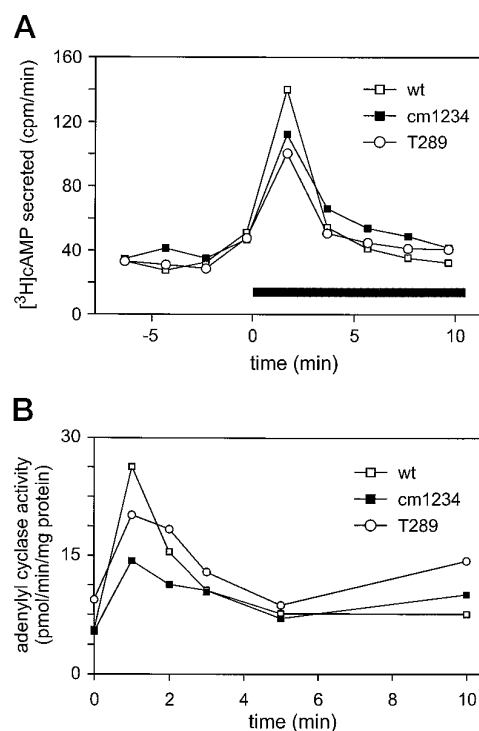


FIG. 6. cAMP-induced adenylyl cyclase activation. The activity of adenylyl cyclase, stimulated by either wild-type cAR1, cm1234, or T289, was measured by two different methods. *Panel A*, the amount of cAMP-stimulated [³H]cAMP secretion was measured by the perfusion method described under "Experimental Procedures." The time period of stimulation of the cells with 100 nM cAMP in DB is indicated by a gray bar. At other times, the cells were perfused with DB lacking cAMP. *Panel B*, adenylyl cyclase activation trap assay (see "Experimental Procedures"). *car1*[−]/*car3*[−] cells expressing wild-type cAR1 or mutant receptors were stimulated with 100 nM cAMP. At the indicated times, aliquots of cells were rapidly lysed through filters, and adenylyl cyclase activity was immediately measured in the lysates.

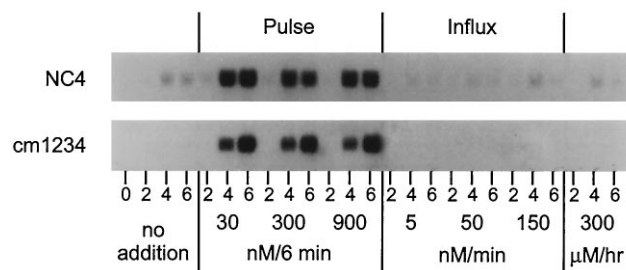


FIG. 7. cAMP-induced gene expression in cm1234 cells. Wild-type cells (NC4) and *car1*[−]/*car3*[−] cells expressing cm1234 harvested during growth phase were incubated in phosphate buffer and stimulated for 6 h with different cAMP regimes as indicated in the figure. mRNA was isolated after 0, 2, 4, and 6 h of incubation, and Northern transfers were probed with a ³²P-labeled cDNA for the contact site A (csA) gene (a gift from A. Noegel).

rates of 5, 50, and 150 nM/min all led to suppression. These results illustrate that cAR1 phosphorylation is not necessary for this adaptation process.

DISCUSSION

We have demonstrated that mutant receptors that cannot undergo agonist-mediated phosphorylation can support development of *Dictyostelium*. In addition, they can elicit multiple responses including chemotaxis, activation of adenylyl and guanylyl cyclases, and actin polymerization. Our observations show that the tightly regulated adaptation of all of these responses does not depend on receptor phosphorylation. Thus, we have discovered that a mechanism(s) other than receptor phos-

phorylation terminates multiple chemoattractant receptor-mediated responses.

This result was surprising in view of findings in other systems that implicate agonist-induced receptor phosphorylation in termination of a variety of responses. In transgenic mice, a truncated form of rhodopsin was found to mediate abnormally prolonged hyperpolarizations, suggesting that phosphorylation is required for the normal termination of responses to light (27). Moreover, numerous studies have indicated a role for β -adrenergic receptor phosphorylation in its uncoupling from G-protein and sequestration (6, 28). In addition, *Saccharomyces cerevisiae* cells expressing truncated pheromone receptors exhibit heightened pheromone sensitivity (29).

Receptor phosphorylation might be expected to play a role in background subtraction during chemotaxis. Such a mechanism could account for the ability of wild-type cells to sense and move up shallow gradients of chemoattractant superimposed on a constant subsaturating level of stimulus (30). If cAR1 phosphorylation was essential for this sensitivity adjustment, then chemotaxis mediated by the mutant receptors would be expected to be grossly impaired. Our results show that non-phosphorylatable mutants can mediate chemotaxis over a broad range of agonist concentrations similar to wild-type cells. Thus, background subtraction must be mediated by a phosphorylation-independent mechanism. The range of chemoattractant concentrations to which the mutant receptors can respond is narrower as shown in Fig. 3B. This may reflect their inability to undergo the reduction of receptor affinity caused by phosphorylation.

The putative phosphorylation-independent adaptation mechanism required to explain our findings could theoretically act on any site within the signal transduction pathway. Loss of ligand binding, a rapid ligand-induced reduction in cAR1 affinity, is clearly not the adaptation mechanism as cm1234 and T289 are severely impaired in this phenomenon. Other agonist-induced covalent modifications of the receptor or agonist-induced conformational change of the receptor could act as the phosphorylation-independent adaptation mechanism that we uncovered. Alternatively, a cytosolic protein might compete with the G-protein for binding to the ligand-occupied receptors. The receptor kinase itself or an arrestin might play this role. G2, the G-protein-coupled to cAR1, is known to be phosphorylated upon stimulation of cells with ligand (31). However, this phosphorylation is unlikely to be the primary cause of adaptation as it is transient, and the phosphorylation site in the Ga2 subunit can be eliminated without phenotypic consequence (32).

Mounting evidence suggests that a distal component in the adenylyl cyclase pathway is a target of adaptation. GTP γ S, which can activate adenylyl cyclase in lysates of sensitive cells, fails to do so in lysates of adapted cells (33). This is correlated with a reduction in the ability of adapted cell membranes to bind CRAC, a cytosolic protein essential for adenylyl cyclase activation (34). Thus, the receptor-mediated regulation of membrane binding sites for CRAC likely plays an important role in adaptation of the adenylyl cyclase response. The existence of a pleckstrin homology domain in CRAC and its inability to bind membranes of G β null cells suggest that G $\beta\gamma$ dimers are a part of the binding site for CRAC and a possible target of the regulatory modification that results in adaptation (35).

Although our findings reveal the existence of a phosphorylation-independent adaptation mechanism, they do not exclude the possibility that this mechanism and receptor phosphorylation are functionally redundant. Our preliminary observation that *deadaptation* of the adenylyl cyclase response is more

rapid in cm1234 cells than in wild-type cells is consistent with this possibility and suggests that receptor dephosphorylation normally limits the rate of recovery from the adapted state. Further investigation will be required to clarify this possibility.

Could higher eukaryotic systems possess a similar phosphorylation-independent mechanism of adaptation? Evidence for such a mechanism has not been observed in the studies of rhodopsin and β -adrenergic receptors. It might possibly exist in these systems and could have been overlooked as a result of the *in vitro* approaches taken, or in the case of β -adrenergic receptors, the cultured cells chosen for heterologous expression. It is also possible that different receptors utilize different mechanisms of adaptation. While rhodopsin and β -adrenergic receptor-mediated responses might be regulated exclusively by receptor phosphorylation, other mammalian G-protein-coupled receptors, such as chemokine receptors, might be regulated by phosphorylation-independent mechanisms like those our studies have revealed in *Dictyostelium*.

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